IAI
Journals.ASM.org

Polyclonal Mucosa-Associated Invariant T Cells Have Unique Innate Functions in Bacterial Infection

Wei-Jen Chua,[a] Steven M. Truscott,[b] Christopher S. Eickhoff,[b] Azra Blazevic,[b] Daniel F. Hoft,[b] and Ted H. Hansen[a]

Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri, USA,[a] and Division of Infectious Diseases, Allergy and Immunology, Department of Internal Medicine, Saint Louis University Medical Center, St. Louis, Missouri, USA[b]

Mucosa-associated invariant T (MAIT) cells are a unique population of αβ T cells in mammals that reside preferentially in mucosal tissues and express an invariant Vα paired with limited Vβ T-cell receptor (TCR) chains. Furthermore, MAIT cell development is dependent upon the expression of the evolutionarily conserved major histocompatibility complex (MHC) class Ib molecule MR1. Using *in vitro* assays, recent studies have shown that mouse and human MAIT cells are activated by antigen-presenting cells (APCs) infected with diverse microbes, including numerous bacterial strains and yeasts, but not viral pathogens. However, whether MAIT cells play an important, and perhaps unique, role in controlling microbial infection has remained unclear. To probe MAIT cell function, we show here that purified polyclonal MAIT cells potently inhibit intracellular bacterial growth of *Mycobacterium bovis* BCG in macrophages (MΦ) in coculture assays, and this inhibitory activity was dependent upon MAIT cell selection by MR1, secretion of gamma interferon (IFN-γ), and an innate interleukin 12 (IL-12) signal from infected MΦ. Surprisingly, however, the cognate recognition of MR1 by MAIT cells on the infected MΦ was found to play only a minor role in MAIT cell effector function. We also report that MAIT cell-deficient mice had higher bacterial loads at early times after infection compared to wild-type (WT) mice, demonstrating that MAIT cells play a unique role among innate lymphocytes in protective immunity against bacterial infection.

Innate and adaptive components of the cellular immune system function sequentially to provide protection against microbial infection. Unlike conventional CD4 and CD8 T cells, which must undergo clonal expansion in response to infection, NK cells expressing germ line-encoded receptors respond rapidly to kill infected cells or release cytokines and provide the initial defense during infection. To kinetically bridge the innate and adaptive responses, innate T cells, such as invariant natural killer T (iNKT) cells and M3-restricted T cells, respond more quickly than conventional T cells to infection. Innate T cells are less dependent upon clonal expansion than conventional CD4 and CD8 T cells, because they appear to detect molecular patterns or danger signals rather than specific peptides bound to polymorphic major histocompatibility complex (MHC) molecules. For example, iNKT cells use an invariant T-cell receptor alpha (TCRα) chain (Vα24-Jα18 in humans and the homologous Vα14-Jα18 in mice) combined with a limited set of TCRβ chains to detect the nonclassical MHC class I (class Ib) molecule CD1d, which binds an assortment of self and microbial lipid antigens. However, iNKT cells display little antigen discrimination, since the invariant TCRα chain primarily engages the CD1d-lipid complex while the TCRβ chain makes limited contact (6). Additionally, iNKT cell activation can occur by various pathways, some of which do not require the iNKT detection of CD1d (8, 74). Therefore, with a limited TCR repertoire, iNKT cells can rapidly secrete cytokines in response to a wide range of infections by bacterial, viral, protozoan, or fungal pathogens. The immunomodulatory role of iNKT cells is well established, but the physiological role of iNKT cells in host immunity remains incompletely understood (4, 69). Mucosa-associated invariant T (MAIT) cells, a recently identified T-cell subset in mammals, share striking similarities and interesting differences with iNKT cells (73). MAIT cells have been implicated recently in detection of diverse microbial infections (33, 50), raising important questions of whether and how they might function in pathogen protection as an addition to the innate T-cell population.

Unlike iNKT cells, MAIT cells preferentially reside in mucosal tissues, and their homotypic expansion is dependent upon commensal microbes (72). In addition, MAIT cells are more abundant in humans than mice, whereas iNKT cells are more abundant in mice than humans (73). Similar to iNKT cells, MAIT cells express an invariant TCR that recognizes a novel nonpolymorphic class Ib molecule. More specifically, MAIT cells express an invariant TCRα chain (Vα7.2-Jα 33 in humans and the homologous Vα19-Jα33 in mice) that is preferentially associated with certain TCRβ chains (Vβ2, Vβ13, and Vβ22 in humans and Vβ6 and Vβ8 in mice) (45, 71). Most mouse MAIT cells are CD4/CD8 double negative (DN), but some are also singly positive for CD8 or CD4 coreceptors (45, 55, 72). Human MAIT cells have been reported in either the DN or $CD8^+$ subsets (33, 55, 71). The developmental selection and peripheral activation of MAIT cells is restricted by the nonpolymorphic class Ib molecule MHC-related protein 1 (MR1) (72). The selection of MAIT cells in the thymus requires the expression of MR1 on hematopoietic cells, likely including double-positive (DP) thymocytes, macrophages (MΦ), and dendritic cells (DCs) (13, 55, 72). The *Mr1* gene is highly conserved

Received 16 March 2012 Returned for modification 18 April 2012
Accepted 28 June 2012
Published ahead of print 9 July 2012
Editor: J. L. Flynn
Address correspondence to Ted H. Hansen, hansen@wustl.edu, or Daniel F. Hoft, hoftdf@slu.edu.
Supplemental material for this article may be found at http://iai.asm.org/.

doi:10.1128/IAI.00279-12

among mammals (64, 78, 79). The predicted amino acid sequences of mouse and human MR1 are 89%/90% identical in their α1/α2 domains. In contrast, mouse and human CD1 α1/α2 domains are only 60%/62% identical (38). Interestingly, the sequence analysis of *Mr1* gene orthologs has indicated their strong purifying selection during evolution, suggesting their physiological importance (41). In functional support of this conclusion, mouse and human MAIT cells, like iNKT cells, have the ability to be activated in a cross-species manner (41).

The notion that MR1 presents antigen to MAIT cells is supported by extensive mutagenesis studies (39, 56) as well as by the fact that all αβ T cells characterized thus far detect MHC or MHC-like molecules bound by antigen. What is known about the MR1 presentation pathway is that MAIT cell development and activation is independent of transporters associated with antigen processing (TAP) (40, 71). Moreover, the activation of mouse MAIT cell hybridomas by MR1-overexpressing cell lines was found to be proteasome independent and enhanced by endocytic trafficking (40). Thus, MR1 clearly does not use the same antigen presentation pathway as classical MHC class I (class Ia) molecules. More interestingly, unlike class Ia or CD1 molecules, which are constitutively expressed on the plasma membranes of antigen-presenting cells (APCs), MR1 proteins reside mostly in the endoplasmic reticulum (ER), and only low levels have been detected at the plasma membrane (13, 35, 40). However, surface expression of mouse MR1 can be stabilized at the cell surface using selective monoclonal antibodies (MAbs) (13), whereas human MR1 was shown to be inducible on a human lung epithelial cell line after *in vitro* infection with *Mycobacterium tuberculosis* (33). Collectively, these findings demonstrate that MR1 activation of MAIT cells represents a novel and unique mechanism of αβ T-cell activation that has been conserved throughout mammalian evolution, suggesting physiologic importance in host immunity.

In support of their physiologic importance, two recent studies showed that human and mouse MAIT cells can detect microbial infection in an MR1-restricted manner (33, 50). In a human study, Gold et al. cloned *M. tuberculosis*-reactive $CD8^+$ T cells from infected and uninfected individuals and found in both populations that a high frequency of the *M. tuberculosis*-reactive T cells are human MR1-restricted MAIT cells (33). Furthermore, these *M. tuberculosis*-reactive human MAIT cells are also activated by APCs infected with other bacterial strains, including *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus* but not *Listeria monocytogenes*. A concomitant study by Le Bourhis et al. showed that both mouse and human MAIT cells display activation markers after *in vitro* coculture with APCs infected with various strains of bacteria and yeasts but not viral pathogens (50). This highly cross-reactive recognition of microbial-infected APCs by MAIT cells suggests that their activation may be more similar to pattern recognition than ligand discrimination. However, the upregulation of activation markers on MAIT cells was found not to be dependent upon known innate receptors or adaptors, including Toll-like receptors (TLRs), Nod-like receptors, MyD88, and TRIF (50). In contrast, innate receptor pathways have been shown to be important in iNKT cell activation both *in vivo* and *in vitro* (8, 9, 15, 59, 74, 75). Regarding the clinical relevance of MAIT cells, the two studies mentioned above reported that patients with ongoing *M. tuberculosis* infection have fewer MAIT cells in the blood and higher levels in the lung than healthy individuals (33, 50). This observation suggested that MAIT cells could migrate from the periphery into infected tissues to provide protection during infection.

To probe the role of MAIT cells in infection *in vivo*, antibacterial immune responses have been compared in MR1-deficient ($MR1^{-/-}$) and wild-type (WT) mice; however, findings using this approach thus far have been ambiguous, complicating mechanistic interpretations. For example, Le Bourhis et al. reported that there was no significant difference in bacterial burden between $MR1^{-/-}$ and WT mice infected with *Mycobacterium abscessus* (50). This finding was discordant with their observation that *M. abscessus* activated MAIT cells *in vitro*. In a subsequent study, Georgel et al. reported that MR1 was not required for bacterial clearance after infection with *E. coli*, *Shigella dysenteriae*, or *Yersinia enterocolitica*, but MAIT cells were required for clearance of *Klebsiella pneumoniae* (32). It is unclear why *in vitro* activation experiments showed that mouse MAIT cells respond to APCs infected with diverse microbes, yet most *in vivo* experiments showed that $MR1^{-/-}$ mice which lack MAIT cells are not impaired in their ability to control infection with most bacterial strains tested. A possible explanation of this conundrum is that WT mice lack a sufficient number of MAIT cells to show an antibacterial phenotype. Consistent with this explanation, Vα19-Jα33 TCR-transgenic mice that overexpress MAIT cells had lower bacterial loads than control mice after infection with *E. coli* or *M. abscessus* (50). However, because the TCR-transgenic mice used in these comparisons have few if any conventional $CD4^+$ and $CD8^+$ T cells, this model system lacks physiologic context or the ability to address the question of whether MAIT cells have a nonredundant role in controlling bacterial infection (12). Another possible explanation to the inconsistency between *in vivo* and *in vitro* studies is that perhaps an infection model in mucosal tissues, where MAIT cells mainly reside, needs to be employed. To our knowledge, *in vivo* infections that have been reported so far to compare $MR1^{-/-}$ and WT mice are conducted through intraperitoneal injection (32, 50). The *in vivo* role of MAIT cells may be overlooked in the systemic challenge and therefore did not provide results that correlated with the *in vitro* reactivity to diverse microbes.

To determine whether MAIT cells function as innate T cells and what unique role they might play in bacterial infection, we employed *in vitro* and *in vivo* assays and identified a robust phenotype. Using the *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) infection *in vitro*, we showed that MAIT cells secrete gamma interferon (IFN-γ), which enhances the antibacterial activity of infected MΦ in a manner dependent upon IL-12/23p40. Unexpectedly, the antibacterial activity in these *in vitro* assays was found to be largely independent of MR1 expression by infected MΦ, even though MR1 expression is required during ontogeny for MAIT cells to acquire their innate function. Furthermore, our *in vivo* studies using low-dose aerosol challenge showed that $MR1^{-/-}$ mice had significantly higher bacterial loads in the lung after mycobacterial infection than WT mice. These findings demonstrate that the antibacterial activity of MAIT cells is unique and not redundant with the activities of other innate T-cell populations in the immune system.

MATERIALS AND METHODS

Mice. C57BL/6 and IL-12/23p40$^{-/-}$ mice were purchased from The Jackson Laboratory. $MR1^{-/-}$, Vα19*i*TgMR1$^{+/+}$, and Vα19*i*TgMR1$^{-/-}$ mice (all C57BL/6 background) were gifts from S. Gilfillan (Washington University). The Vα19*i* transgene in these mice is the canonical TCR Vα of

MAIT cells shared by >90% of mouse MAIT cells, and MAIT cells isolated from the transgenic mice used in this study have limited endogenous TCR Vβ usage and surface marker characteristics of polyclonal MAIT cells (45). Mice were bred and maintained under specific-pathogen-free conditions and were used by protocols in the studies approved by the Animal Studies Committee of Washington University.

Antibodies and cytokine. Anti-MR1 MAbs 26.5 and 8F2.F9 were reported previously (13, 39) and used as a mixture in the *in vitro* cultures. Anti-IFN-γ (H22) was a gift from R. Schreiber (Washington University). Anti-IL-12/23p40 MAb (C17.8) was purchased from R&D Systems (Minneapolis, MN). Anti-IL17A (18H10) was purchased from Mabtech (Mariemont, OH). All antibodies were used at the concentration of 10 μg/ml in the *in vitro* cultures. Recombinant mouse IL-12 was purchased from Peprotech (Rocky Hill, NJ) and reconstituted according to the instructions.

T-cell isolation. Spleen cells from naïve mice were aseptically removed and used as the source for T-cell isolation. A single-cell suspension was prepared, and red blood cells were lysed with ammonium chloride. Cells were washed, counted, and subjected to magnetic labeling and separation according to the instructions of the pan-T-cell isolation kit II (130-095-130; Miltenyi Biotec, Auburn, CA). The enriched T cells were used in the *in vitro* cultures.

***In vitro* analysis of the inhibition of intracellular *M. bovis* BCG growth in MΦ by T cells.** The procedures for analyzing the inhibition of intracellular *M. bovis* BCG growth in MΦ by T cells, including the *in vitro* culture of bone marrow-derived MΦ, the infection of mycobacteria, the coculture of MΦ and T cells, and the measurement of intracellular bacterial growth, were adopted and modified from published reports and protocols (18, 26, 61, 77). In brief, bone marrow was flushed from femurs of mice with D10 medium, which is Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine (Mediatech, Manassas, VA), 10 mM HEPES buffer (Mediatech, Manassas, VA), 1× nonessential amino acids (Mediatech, Manassas, VA), 1 mM sodium pyruvate (Mediatech, Manassas, VA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Red blood cells were lysed, and the remaining bone marrow cells were washed and plated at 2×10^4 viable cells per well in 96-well plates (flat bottom) in D10 medium supplemented with 20 ng/ml recombinant murine macrophage colony-stimulating factor (rmM-CSF; 315-02; Peprotech, Rocky Hill, NJ). Cells were incubated at 37°C in 5% CO_2. After 3 days, the attached cells were gently washed with warm antibiotic-free $D10^-$ medium (D10 medium without penicillin and streptomycin). The attached cells were cultured in $D10^-$ medium containing fresh rmM-CSF (20 ng/ml) for an additional 4 days at 37°C in 5% CO_2. After the 7-day culture period, bone marrow-derived MΦ formed a confluent monolayer, and the density of MΦ was estimated to be 2×10^5 cells per well in 96-well plates. MΦ were infected with *M. bovis* BCG (Danish strain) at a multiplicity of infection (MOI) of 3:1 (bacteria to MΦ) in $D10^-$ medium at 37°C in 5% CO_2. After overnight culture, infected MΦ were washed with $D10^-$ medium to remove the extracellular *M. bovis* BCG before coculture with purified T cells. Purified T cells were obtained as described above and added to the wells of infected MΦ at the ratio of 1:1 in $D10^-$ medium at 37°C in 5% CO_2 for 72 h. At the end of coculture, supernatants were collected for the measurement of the production of cytokines and nitric oxide. Cells were lysed with 0.2% saponin to release intracellular *M. bovis* BCG. Mycobacteria were radiolabeled with tritiated uridine as previously described (77) to determine the viability of bacteria under different coculture conditions. Briefly, [5,6-^{3}H]uridine (Perkin Elmer, Waltham, MA) at 1 μCi/ml prepared in Middlebrook 7H9 broth supplemented with 10% ADC enrichment medium (BD BBL 211887; Becton, Dickinson, Franklin Lakes, NJ) was added to saponin lysates. After incubation at 37°C for 72 h, the tritiated uridine-pulsed mycobacteria were harvested (Tomtec Harvester 96 MACH III M; Tomtec, Hamden, CT) onto glass fiber filters (Perkin Elmer, Waltham, MA), and radioactivity was quantitated by liquid scintillation counting (Wallac MicroBeta TriLux 1450; Perkin Elmer, Waltham, MA). The percentages of *M. bovis* BCG growth inhibition under different coculture conditions were calculated by the following formula: percent inhibition = 100 − [100 × (disintegrations per minute from T cell-infected MΦ cocultured wells/disintegrations per minute from infected MΦ alone wells)].

Measurement of cytokine and nitric oxide (NO) production. Culture supernatants were collected after 72 h of coculture. The concentration of IFN-γ and IL-17A was quantified by standard sandwich ELISAs according to the manufacturer's instruction (R&D Systems, Minneapolis, MN). NO production was measured by the Griess reaction, using commercial Griess reagent (G4410; Sigma-Aldrich, St. Louis, MO). Serially diluted $NaNO_2$ (S-2252; Sigma-Aldrich, St. Louis) (0 to 100 μM) was used to generate a standard curve to quantify the amount of nitrite (NO_2^-) in supernatants (37, 57, 62).

***In vivo* infection of *M. bovis* BCG.** WT and $MR1^{-/-}$ mice were infected with *M. bovis* BCG (Danish strain) via aerosol or the intranasal route. In the aerosol infection, *M. bovis* BCG was diluted to 10^7 CFU/ml in phosphate-buffered saline (PBS) with 0.04% Tween 80. A nebulizer (bioaerosol nebulizing generator [BANG]; CH Technologies) was set to liquid feed at 1 ml/min and airflow at 1 liter/min to expose mice to *M. bovis* BCG. A 20-min exposure to the aerosol resulted in the delivery of 100 to 300 infectious bacilli per mouse lung. In the intranasal infection, WT and $MR1^{-/-}$ mice were injected with *M. bovis* BCG via the nares (10^7 CFU per mouse) while mice were under anesthesia and laid on their dorsal side on a plastic mouse holder (60). On day 10 and 30 postinfection, the lungs were harvested and homogenized. To determine the mycobacterial colonization, the lung homogenate was serially diluted (1:10 and 1:100) and plated on Middlebrook 7H10 agar plates supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) enrichment medium (BD BBL 212240; Becton, Dickinson, Franklin Lakes, NJ). Two to three weeks later, mycobacterial CFU had formed on the agar plates and were counted.

***Ex vivo* stimulation of bronchoalveolar lavage (BAL) cells by mycobacterial antigens and IFN-γ enzyme-linked immunosorbent spot (ELISPOT) analysis.** To determine the IFN-γ response elicited in the lung by *M. bovis* BCG infection, bronchoalveolar lavage (BAL) cells from WT and $MR1^{-/-}$ mice were harvested. BAL cells from the same genotype were pooled and used in the ELISPOT assay. The BAL cells were stimulated *ex vivo* with *M. bovis* BCG (MOI = 3) or various antigens derived from *M. tuberculosis*, including culture filtrate proteins (CF) (10 μg/ml; Colorado State University, Fort Collins, CO), cell wall (CW) fraction (50 μg/ml; Colorado State University, Fort Collins, CO), and the Ag85B peptide pool (Ag85Bpp) (2 μg/ml; JPT Peptide Technologies GmbH, Berlin, Germany). The frequency of IFN-γ-producing cells in the BAL cells was measured by ELISPOT analysis. Anti-IFN-γ MAbs, clones AN18 and R4-6A2 (biotinylated), were purchased from eBioscience (San Diego, CA) and used as a pair to capture the release of IFN-γ of BAL cells. Streptavidin-alkaline phosphatase (ALP) (Mabtech, Mariemont, OH) and BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium) substrate (Fisher Scientific, Pittsburgh, PA) were used to visualize the IFN-γ spot-forming units (SFUs). A CTL-ImmunoSpot S6 microanalyzer and ImmunoSpot professional software (Shaker Heights, OH) were used to quantitate the SFUs.

RESULTS

MR1-restricted MAIT cells have an innate function and inhibit mycobacterial growth in MΦ. To define the potential role of MAIT cells in controlling bacterial infection, we established an *in vitro* infection assay whereby purified MAIT cells were cocultured with *M. bovis* BCG-infected MΦ. In these coculture assays, purified transgenic MAIT cells composed of 50% of DN, 25% $CD8^+$, and 25% $CD4^+$ T cells (45) (data not shown) from naïve Vα19*i*TgMR1$^{+/+}$ mice were used. As a negative control, purified naïve T cells from Vα19*i*TgMR1$^{-/-}$ mice were included (45). In these mice, T cells that express the Vα19-Jα33 transgene are found in the periphery, but due to the lack of selection by MR1 during

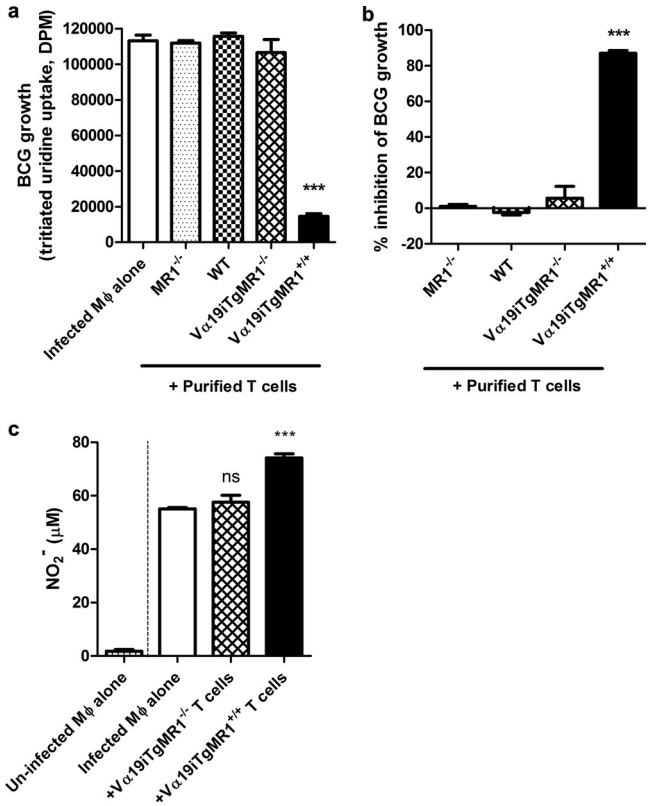

FIG 1 Innate MR1-restricted MAIT cells inhibit mycobacterial growth in MΦ. (a and b) *M. bovis* BCG-infected MΦ derived from WT mice were cultured alone or cocultured with naïve T cells from MR1$^{-/-}$, WT, Vα19iTgMR1$^{-/-}$, or Vα19iTgMR1$^{+/+}$ mice for 72 h. The intracellular growth of mycobacteria in MΦ was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute) (a). (b) The percentage of mycobacterial growth inhibition was assessed from radioactivity in panel a to demonstrate the extent of suppression of mycobacterial growth by naïve T cells from different mice. See Materials and Methods for the formula used in the calculation of the percentage. (c) Nitric oxide (NO) production by uninfected MΦ, infected MΦ, or infected MΦ cocultured with naïve T cells (Vα19iTgMR1$^{-/-}$ or Vα19iTgMR1$^{+/+}$). Supernatants were collected after 72 h of culture. The amount of nitrite (NO_2^-) in supernatants was measured. Results are means ± standard errors of the means (SEM) of the triplicate of different culture conditions. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison posttest comparing every culture condition (***, $P \leq 0.001$; ns, not significant). The dotted line in panel c indicates that the uninfected MΦ were not included in statistical comparison. Data are results from one of three independent experiments.

development, these T cells do not display MAIT cell characteristics, including cytokine secretion, effector/memory phenotype, and bias in the TCRβ usage (45, 55). For comparisons, we also included naïve T cells from WT mice that have a very low frequency of MAIT cells and from MR1$^{-/-}$ mice that lack detectable MAIT cells (72). These various populations of naïve T cells were cocultured with *M. bovis* BCG-infected MΦ. As shown in Fig. 1a and b, MAIT cells inhibited the intracellular growth of *M. bovis* BCG in MΦ by 40 to 80% (varying in several independent experiments) compared to bacterial growth in MΦ in the absence of MAIT cells. Importantly, control transgenic T cells from Vα19*i*TgMR1$^{-/-}$ mice showed no inhibition of bacterial growth in *M. bovis* BCG-infected MΦ (Fig. 1a and b). Thus, the antibacterial function of Vα19-Jα33 TCR-bearing T cells requires MR1 selection during development. T cells from naïve MR1$^{-/-}$ and WT mice also failed to inhibit the intracellular growth of *M. bovis* BCG in MΦ, suggesting that normal levels of MAIT cells or other innate T cells are not sufficient to control the mycobacterial growth in these coculture assays (Fig. 1a and b). In addition, MAIT cells, but not control transgenic T cells, enhanced nitric oxide (NO) production by infected MΦ (Fig. 1c), indicating an increase in microbicidal activity of MΦ and providing further evidence of the ability of MAIT cells to control bacterial infection.

MAIT cells are a potent source of innate IFN-γ. IFN-γ is a key cytokine for optimal antimycobacterial immunity (16, 28, 29). In the early stage of infection, innate T cells and NK cells are activated by mycobacterium-infected MΦ to release IFN-γ (27, 49, 66). This innate source of IFN-γ is indispensable for enhancement in bactericidal activity of MΦ, including the induction of inducible nitric oxide synthase (iNOS) (54). It has been shown that human MAIT cells produce IFN-γ in response to mycobacterium-infected APCs (33, 50). However, whether IFN-γ production is required for MAIT cells to control mycobacterial growth was not previously investigated. To address this question, we first determined whether mouse MAIT cells secrete IFN-γ when cocultured with *M. bovis* BCG-infected MΦ. As shown in Fig. 2a, MAIT cells responding to infected MΦ had high levels of IFN-γ production. In contrast, the supernatant from the cocultures of Vα19*i*TgMR1$^{-/-}$, WT, or MR1$^{-/-}$ T cells showed low, if any, IFN-γ production (Fig. 2a). To test whether IFN-γ production by MAIT cells was required for the control of mycobacterial growth, anti-IFN-γ blocking antibody was added to the coculture. Anti-IFN-γ antibody ablated the inhibition of bacterial growth by MAIT cells (Fig. 2b) and also suppressed the enhancement of NO production by MAIT cells (Fig. 2c). These findings suggested that MAIT cells are a potent source of innate IFN-γ which is required for their ability to control mycobacterial growth in MΦ.

Activated MAIT cells produce IL-17A in response to infection. In addition to IFN-γ, MAIT cells have also been reported to produce IL-17 in response to mitogenic or cytokine stimulations (10, 25). These finding raise the question of whether MAIT cells secrete IL-17 when encountering bacterium-infected APCs. As shown in Fig. 3a, MAIT cells produced high levels of IL-17A when cocultured with *M. bovis* BCG-infected, but not uninfected, MΦ. To determine whether IL-17A secretion was required for MAIT cell control of the intracellular growth, an anti-IL-17A blocking antibody was added to the coculture. Interestingly, the blockage of IL-17 in the coculture did not affect the ability of MAIT cells to control the intracellular growth of *M. bovis* BCG in MΦ (Fig. 3b). The combined results (Fig. 2 and 3) suggest that during infection, MAIT cells produce IFN-γ to provide direct control of bacterial growth in infected APCs, whereas their IL-17 production may help stimulate the adaptive immune response (see below).

Effector function of MAIT cells is dependent on the innate signal IL-12 from infected MΦ. Next, we sought to dissect the mechanism by which APCs activate MAIT cells during infection. iNKT cells can receive two signals from APCs during infection (8, 74). One signal is the cognate interaction between CD1d/lipid complexes on APCs and TCRs on iNKT cells (6), whereas the more dominant signal was recently shown to be IL-12 secretion by the microbe-infected APCs (9). Our results have demonstrated that MR1 is required for MAIT cells during development to obtain their effector function (Fig. 1 and 2a; also, see Fig. S1 in the supplemental material). To determine if cognate recognition of MR1

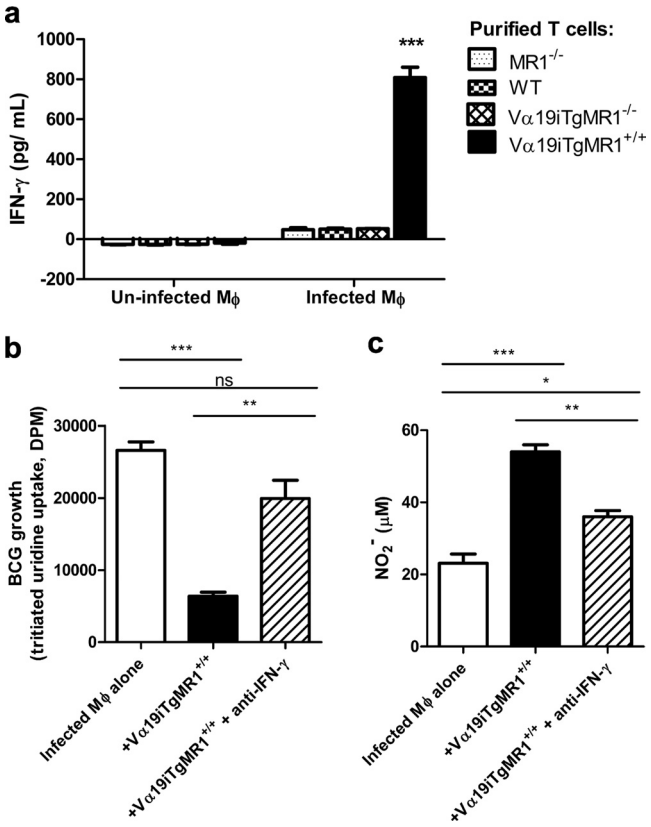

FIG 2 MAIT cells are a potent source of innate IFN-γ enhancing the microbicidal activity of infected MΦ. (a) IFN-γ production by naïve T cells from MR1$^{-/-}$, WT, Vα19iTgMR1$^{-/-}$, and Vα19iTgMR1$^{+/+}$ mice when cocultured with uninfected or *M. bovis* BCG-infected MΦ. Supernatants were collected after 72 h of coculture. The concentration of IFN-γ in supernatants was measured by ELISA. (b) Effect of IFN-γ blockage on the inhibition of mycobacterial growth by Vα19iTgMR1$^{+/+}$ T cells. *M. bovis*-infected MΦ were cultured alone or cocultured with Vα19iTgMR1$^{+/+}$ T cells in the absence or presence of an anti-IFN-γ MAb for 72 h. The intracellular growth of mycobacteria in MΦ was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute). (c) Effect of IFN-γ blockage on the enhancement in NO production of infected MΦ by Vα19iTgMR1$^{+/+}$ T cells. Supernatants from the cultures used for panel b were collected before cell lysis for the radiolabeling of mycobacteria. The amount of NO_2^- in supernatants was measured. Results are the means ± SEM from triplicate assays of each set of culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison posttest comparing all culture conditions (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant). Data are results from one of three independent experiments.

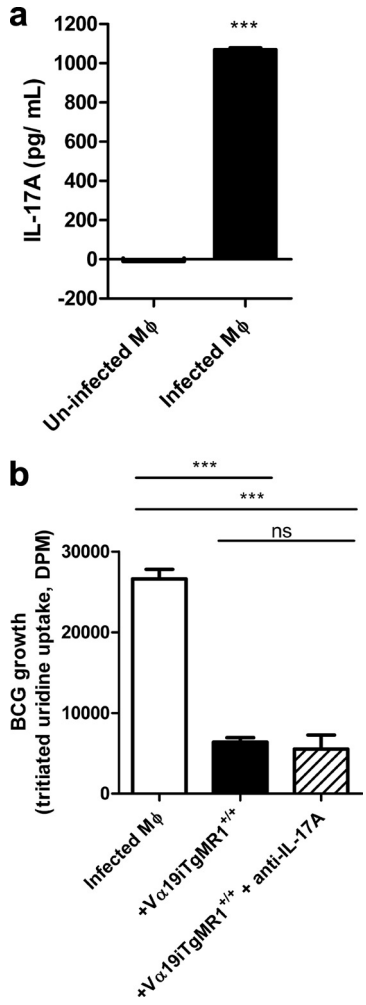

FIG 3 Innate MAIT cells produce IL-17A in response to infection. (a) IL-17A production of Vα19iTgMR1$^{+/+}$ T cells when cocultured with uninfected or *M. bovis* BCG-infected MΦ. Supernatants were collected after 72 h of coculture. The concentration of IL-17A in supernatants was measured by ELISA. (b) Effect of IL-17A blockage on the inhibition of mycobacterial growth by Vα19iTgMR1$^{+/+}$ T cells. *M. bovis* BCG-infected MΦ were cultured alone or cocultured with Vα19iTgMR1$^{+/+}$ T cells in the absence or presence of an anti-IL-17A MAb for 72 h. The intracellular growth of mycobacteria in MΦ was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute). Results are means ± SEM from triplicate assays of each set of culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison posttest comparing all cultures (***, $P \leq 0.001$; ns, not significant). Data are results from one of three independent experiments.

on infected MΦ is required for MAIT cell activation, anti-MR1 blocking MAbs (13, 33, 39, 50) or MR1$^{-/-}$ MΦ were used in the coculture assay. Surprisingly, anti-MR1 MAbs did not block MAIT cell production of IFN-γ and IL-17A (Fig. 4a and c) or enhancement of NO production (Fig. 4e) when cocultured with WT MΦ infected with *M. bovis* BCG. Furthermore, MR1$^{-/-}$ MΦ infected with *M. bovis* BCG elicited MAIT cell responses comparable to those obtained with infected WT MΦ (Fig. 4b, d, and f). Thus, the cognate recognition of MR1 on infected cells was not required for MAIT cytokine responses to infected APCs.

To determine whether the IL-12 signal from infected APCs was responsible for MAIT cell activation, we took advantage of anti-IL-12/23p40 blocking MAb and MΦ from mice deficient in the IL-12/23p40 gene (p40$^{-/-}$). The coculture experiments showed that WT MΦ infected with *M. bovis* BCG failed to activate MAIT cells in the presence of anti-IL-12/23p40 MAb (Fig. 4a, c, and e). Furthermore, p40$^{-/-}$ MΦ infected with *M. bovis* BCG did not elicit IFN-γ or IL-17A production (Fig. 4b and d) or support enhancement of NO production by MAIT cells (Fig. 4f).

We next tested whether MR1-recognition and IL-12 secretion were required for the ability of MAIT cells to control the intracellular bacterial growth in infected MΦ. As shown in Fig. 5a and c, anti-IL-12/23p40 MAb blocked the anti-bacterial activity of MAIT cells, but anti-MR1 MAbs did not (Fig. 5a and c). Furthermore, MAIT cells were not able to control bacterial growth in p40$^{-/-}$ MΦ but were still effective in inhibiting bacterial growth

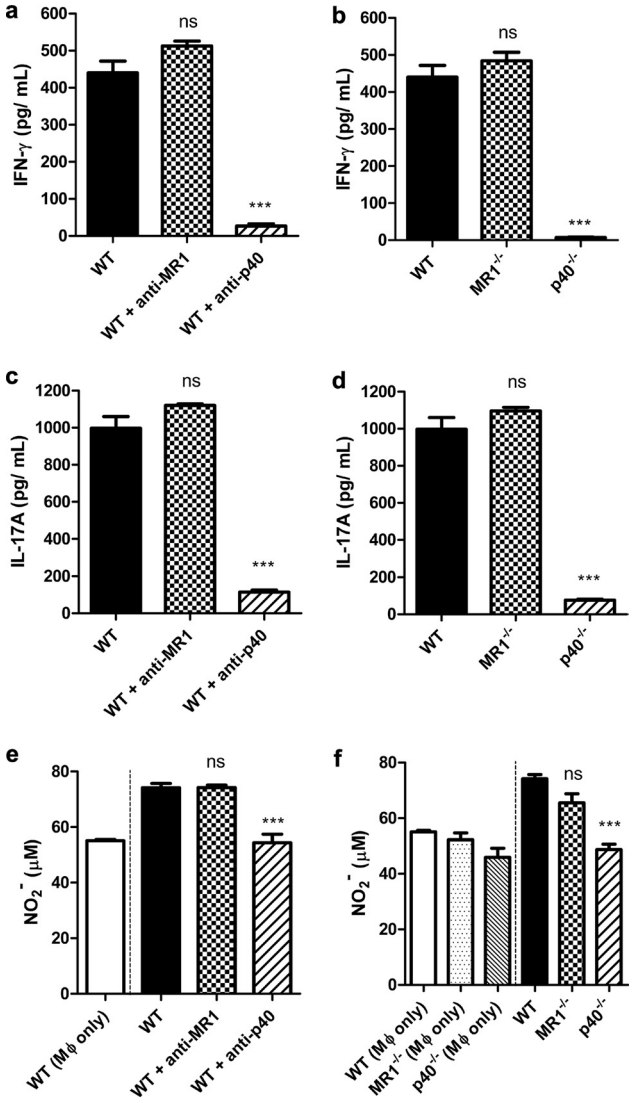

FIG 4 Activation of MAIT cells is dependent on the innate signal IL-12 from infected MΦ. (a, c, and e) MAIT cell activation in the presence of anti-MR1 or anti-IL-12/23p40 MAbs. Vα19iTgMR1$^{+/+}$ T cells were cocultured with *M. bovis* BCG-infected MΦ, and supernatants were collected after 72 h of coculture. The concentrations of IFN-γ (a), IL-17A (c), and NO_2^- (e) in supernatants were measured. Results are means ± SEM from triplicate assays of each set of culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison posttest comparing all cultures. ***, $P \leq 0.001$; ns, not significant (for comparisons to results obtained with MΦ cocultured with Vα19iTgMR1$^{+/+}$ T cells in the absence of blocking MAbs). The dotted line in panel e indicates that the NO production by MΦ cultured alone was not included in the statistical comparison. Data are the results from one of three independent experiments. (b, d, and f) Activation of MAIT cells cocultured with WT, MR1$^{-/-}$, or p40$^{-/-}$ MΦ infected with *M. bovis* BCG. Vα19iTgMR1$^{+/+}$ T cells were cocultured with WT, MR1$^{-/-}$, or p40$^{-/-}$ MΦ infected with *M. bovis* BCG and supernatants, were collected after 72 h of coculture. The concentration of IFN-γ (b), IL-17A (d), and NO_2^- (f) in supernatants were measured. Results are means ± SEM from triplicate assays of each set of culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison posttest comparing all cultures. ***, $P \leq 0.001$; ns, not significant (for comparisons to results obtained with WT MΦ cocultured with Vα19iTgMR1$^{+/+}$ T cells). In panel f, the dotted line indicates that statistical analyses were performed separately for MΦ alone and MΦ cocultured with Vα19iTgMR1$^{+/+}$ T cells. There was no statistical difference in NO production among infected MΦ of different genotypes cultured in the absence of Vα19iTgMR1$^{+/+}$ T cells. Data are results from one of two independent experiments.

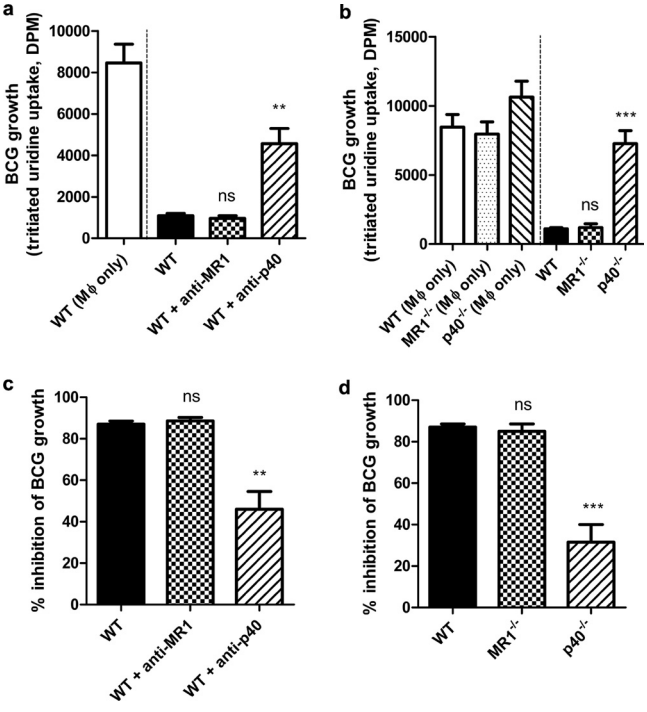

FIG 5 Control of mycobacterial growth by MAIT cells depends on IL-12. (a and c) The inhibition of mycobacterial growth by MAIT cells in the presence of anti-MR1 or anti-IL-12/23p40 MAbs. *M. bovis*-infected MΦ were cultured alone, or cocultured with Vα19iTgMR1$^{+/+}$ T cells in the absence or presence of anti-MR1 MAbs or an anti-IL-12/23p40 MAb for 72 h. The intracellular growth of mycobacteria in MΦ was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute) (a). The percentage of mycobacterial growth inhibition (c) was assessed from radioactivity in panel a to demonstrate the extent of suppression of mycobacterial growth by Vα19iTgMR1$^{+/+}$ T cells in the absence or presence of blocking MAbs. See Materials and Methods for the formula used to calculate inhibition. Results are means ± SEM from triplicate assays of each set of culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison posttest comparing all cultures. **, $P \leq 0.01$; ns, not significant (for comparison to results obtained with MΦ cocultured with Vα19iTgMR1$^{+/+}$ T cells in the absence of blocking MAbs). The dotted line in panel a indicates that MΦ cultured alone were not included in statistical comparison. Data are results from one of three independent experiments. (b and d) Inhibition of mycobacterial growth in WT, MR1$^{-/-}$, or p40$^{-/-}$ MΦ by MAIT cells. WT, MR1$^{-/-}$, or p40$^{-/-}$ MΦ infected with *M. bovis* BCG were cultured alone or cocultured with Vα19iTgMR1$^{+/+}$ T cells for 72 h. The intracellular growth of mycobacteria in MΦ was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute) (b). The percentage of mycobacterial growth inhibition (d) was assessed based on genotype. Results are means ± SEM of the triplicate of different culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison posttest comparing all cultures. ***, $P \leq 0.001$; ns, not significant (for comparison to results obtained with the coculture of infected WT MΦ with Vα19iTgMR1$^{+/+}$ T cells). In panel b, the dotted line indicates that statistical analyses were performed separately for MΦ alone and MΦ cocultured with Vα19iTgMR1$^{+/+}$ T cells. There was no statistical difference in mycobacterial growth among infected MΦ of different genotypes cultured in the absence of Vα19iTgMR1$^{+/+}$ T cells. Data are results from one of two independent experiments.

in MR1$^{-/-}$ MΦ (Fig. 5b and d). The MAIT cell dependence on IL-12 was also revealed by the addition of anti-IL-12/23p40 MAb to the coculture with MR1$^{-/-}$ MΦ infected with *M. bovis* BCG, where MAIT cell activation and effector function were blocked (data not shown). Thus, these *in vitro* findings highlight the im-

portance of MAIT cell control of intracellular bacterial growth in MΦ by a mechanism that is IL-12 dependent and show that in the presence of an innate IL-12 signal, the requirement for cognate engagement of MR1 by MAIT cells was not detected.

The above observations indicated that the IL-12 signal rather than MR1 recognition may dominate MAIT cell activation and effector function in bacterial infection. However, it remained possible that an MR1-restricted interaction between MAIT cells and MΦ may be masked by a strong IL-12 signal. To test this possibility, we added anti-MR1 MAbs to the coculture of MAIT cells and $p40^{-/-}$ MΦ infected with *M. bovis* BCG. As shown in Fig. 6a and b, anti-MR1 MAbs further decreased the already defective production of IFN-γ and IL-17A by MAIT cells in the absence of IL-12 signal from infected MΦ. However, the decrease in MAIT cell cytokine production by anti-MR1 MAbs was not reflected in the control of *M. bovis* BCG growth in $p40^{-/-}$ MΦ (Fig. 6c). These data suggested that the MR1/TCR-mediated signal may be weak and/or transient. IL-12 was shown to amplify weak TCR-mediated activation of iNKT cells (9). The addition of recombinant IL-12 stimulated MAIT cells cocultured with uninfected WT MΦ to release IFN-γ in a dose-dependent manner (Fig. 7). In comparison, control T cells from Vα19*i*TgMR1$^{-/-}$ mice did not respond to recombinant IL-12. We concluded from these mechanistic studies that the innate signal IL-12 is indispensable for MAIT cell function of controlling bacterial infection. In contrast, certain effector functions of MAIT cells may be MR1 independent, in spite of the critical role of MR1 expression during development.

MR1-restricted MAIT cells play a unique role in protective immunity against mycobacterial infection *in vivo*. From our *in vitro* observations, MAIT cells appear to function as innate lymphocytes and are capable of controlling mycobacterial infection. To test the hypothesis that MAIT cells play a nonredundant role in providing immunity against infection, we infected MR1$^{-/-}$ mice with a low dose of *M. bovis* BCG via the aerosol route. At day 10 postinfection, MR1$^{-/-}$ mice showed significantly higher bacterial burdens in the lung than WT mice (Fig. 8a). However, MR1$^{-/-}$ and WT mice showed similar bacterial loads in the lung at day 30 postinfection (Fig. 8a). These results suggest that MAIT cells contribute to controlling bacterial growth at the early stage of infection. To detect a greater cytokine response, we increased the infection dose and delivered *M. bovis* BCG via the intranasal route. Consistent with the result in the aerosol challenge, MR1$^{-/-}$ mice had higher bacterial burden in the lung than WT mice at day 10 after intranasal infection (data not shown). Furthermore, when stimulated *ex vivo* with *M. bovis* BCG and various antigens derived from *M. tuberculosis*, bronchoalveolar lavage (BAL) cells from infected MR1$^{-/-}$ mice showed a lower IFN-γ response than BAL cells from WT mice (Fig. 8b). The antigens tested included culture filtrate proteins (CF) and cell wall (CW) fractions from *M. tuberculosis* H37Rv. Of note, a previous study suggested that the CW fraction of *M. tuberculosis* stimulated $CD8^+$ human MAIT cell clones (33). Thus, the lower IFN-γ response of MR1$^{-/-}$ BAL cells after infection may result from the lack of MAIT cells responding to mycobacterial antigens *ex vivo*. However, it is difficult to interpret these findings without definitive evidence that MR1 binds a ligand and, if so, whether it is microbe derived or an infection-induced self-ligand. Alternatively, BAL cells from infected MR1$^{-/-}$ mice also showed a lower IFN-γ response to Ag85Bpp, the peptide pool of known immunodominant epitopes of Ag85B recognized by $CD4^+$ and $CD8^+$ T cells (1, 44, 53), suggesting a

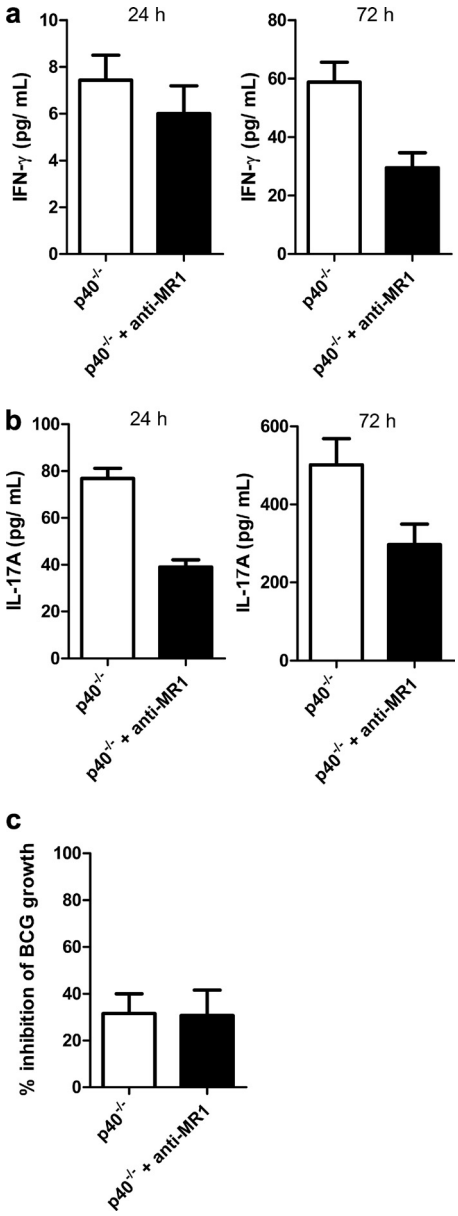

FIG 6 The role of MR1 in MAIT cell activation. (a and b) MAIT cell activation by $p40^{-/-}$ MΦ in the presence of anti-MR1 MAbs. Vα19iTgMR1$^{+/+}$ T cells were cocultured with $p40^{-/-}$ MΦ infected with *M. bovis* BCG in the absence or presence of anti-MR1 MAbs. Supernatants were collected after 24 or 72 h of coculture. (c) The inhibition of mycobacterial growth in $p40^{-/-}$ MΦ by Vα19iTgMR1$^{+/+}$ T cells in the absence or presence of anti-MR1 MAbs. Results are means ± SEM from triplicate assays of each set of culture conditions. Statistical analysis was performed using Student's *t* test. Although there was a trend for cytokine secretion of Vα19iTgMR1$^{+/+}$ T cells to be lower in the presence of MR1 blockage, the difference was not statistically significant. Data are results from two time points in two independent experiments.

poorer adaptive immune response in MR1$^{-/-}$ mice during *M. bovis* BCG infection. Because IL-17A is known to be important for driving T_H1 immunity in the lung after the infections of intracellular pathogens (36, 46, 47, 51), we speculated that the defective IL-17 response in MR1$^{-/-}$ mice due to the lack of MAIT cells results in failure to recruit $CD4^+$ and $CD8^+$ T cells to the site of infection. In fact, BAL cells from infected MR1$^{-/-}$ mice produced

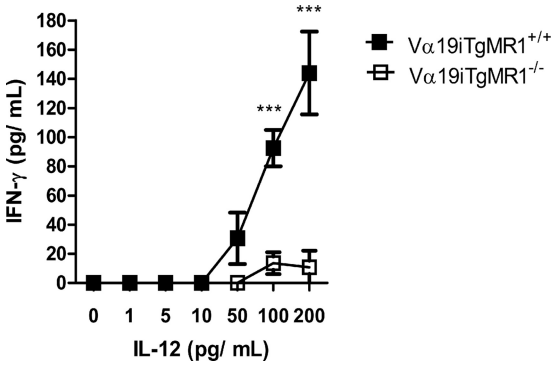

FIG 7 MR1-restricted MAIT cells produce IFN-γ in response to recombinant IL-12 in the absence of infection. Vα19iTgMR1$^{-/-}$ or Vα19iTgMR1$^{+/+}$ T cells were incubated with uninfected WT MΦ in the presence of recombinant IL-12 at various concentrations. Supernatants were collected after 24 h of incubation. The concentration of IFN-γ in supernatants was measured by ELISA. Results are means ± SEM from triplicate assays of each set of culture conditions. Statistical analysis was performed using a two-way ANOVA (***, $P \leq 0.001$). Data are results from one of two independent experiments.

less IL-17A than infected WT mice in response to the CW fraction of *M. tuberculosis* (Fig. 8c). The functional relevance of the published reports that MAIT cells accumulated at the site of infections (33, 50) is supported by the evidence reported here that MAIT cells are a potentially significant source of IFN-γ and IL-17A. In any case, the overall implication of these findings is that MAIT cells not only play a unique role among innate lymphocytes but may also predispose adaptive lymphocytes to mount protective immune responses during infection.

DISCUSSION

In this study, we provide *in vitro* and *in vivo* evidence that MAIT cells exert unique innate functions to control mycobacterial growth. In a coculture assay of purified MAIT cells and mycobacterium-infected MΦ, MAIT cells were found to curtail growth of intracellular bacterial replication associated with the secretion of IFN-γ and IL-17A, known mediators of immunity against intracellular and extracellular bacterial infection (17, 19, 22, 42, 47, 63, 80). Furthermore, the "first-line" production of IFN-γ and IL-17A by innate lymphocytes is known to play an important role in bridging innate and adaptive immunity (3, 21, 36, 46, 51, 67). Although both IFN-γ and IL-17A of MAIT cells were induced during the coculture with mycobacterium-infected MΦ, the control of mycobacterial growth was dependent only upon IFN-γ. Thus, IL-17A secretion by MAIT cells likely has a role other than the direct impact on bacterial intracellular growth in the mycobacterial infection model used here.

Importantly, the antibacterial activity of MAIT cells in our *in vitro* assays was found to be critically dependent upon their *in vivo* development in MR1-positive mice. Although in WT mice MAIT cell development is MR1 dependent, in transgenic mice expressing the MAIT cell canonical Vα19-Jα33 TCRα chain, Vα19*i* T cells can develop in both *Mr1*$^{+/+}$ and *Mr1*$^{-/-}$ mice. However, only MR1-educated Vα19*i* T cells have a MR1-dependent bias in TCRβ chain usage and exhibit effector/memory and cytokine secretion profiles like nontransgenic MAIT cells (45, 55). In complete agreement with this conclusion from previous studies, only Vα19*i* transgenic T cells developing in the MR1-positive mice were found to control intracellular bacterial growth in our *in vitro* assays. Surprisingly, however, these MAIT cells selected by MR1 *in vivo* were found not to rely on MR1 engagement to impair intracellular bacterial replication *in vitro*. Indeed, this conclusion was supported by both the failure of anti-MR1 MAbs to block the antibacterial activity of MAIT cells and the observation that MAIT cells controlled bacterial growth in MR1$^{-/-}$ MΦ. Thus, the MAIT cell effector function *in vitro* did not require TCR recognition of MR1 on infected APCs. In contrast, our observations suggest a noncognate pathway of MAIT cell activation mediated by the innate signal IL-12 during mycobacterial infection. Abolishing the IL-12 signal from infected APCs (with the use of anti-IL-12/23p40 MAb or p40$^{-/-}$ MΦ) diminished the effector function of MAIT cells, including IFN-γ and IL-17A production as well as the inhibition of mycobacterial growth. Of note, when the IL-12 signal was prevented, anti-MR1 MAbs partially inhibited the anti-bacterial activity of MAIT cells, suggesting that an MR1-dependent component may be masked by a strong innate IL-12 signal.

Interestingly, besides infection, MAIT cells have been reported to play a regulatory role in mouse models of autoimmunity (10, 20, 58, 68). For example, Croxford et al. showed that MAIT cells provide protection in murine autoimmune encephalomyelitis (EAE) by inducing the IL-10 production by B cells (20). However, this protective mechanism was shown to be MR1 independent and partly ICOS dependent. More recently, Chiba et al. showed that MAIT cells adoptively transferred into MR1$^{-/-}$ mice augment collagen antibody-induced arthritis (10). To explain how MAIT cells modulate the immune response in the absence of MR1, their *in vitro* analyses showed that MAIT cells are stimulated to proliferate by IL-1β and to produce IL-17A by IL-23 in the absence of TCR signals (10). In the present study, MAIT cells were found to release IFN-γ by the stimulation of IL-12 in the absence of infection. In accordance with these examples of cytokine driven activation, human MAIT cells express high levels of IL-12Rβ2 and IL-23R (2, 5). These combined findings point toward a model in which, after selection by MR1 in thymus, MAIT cells are programmed to respond to innate signals, at least some of which can occur without concurrent MR1 recognition. Alternatively, after egress from the thymus, MAIT cells adapt by quickly responding to infection (34). During infection, MR1 on either APCs or MAIT cells themselves may be a sufficient signature to allow MAIT cell activation by an innate cytokine signal.

Despite the above evidence of the non-MR1-dependent functions of MAIT cells, previously published studies have provided evidence for cognate interaction between MR1 and MAIT cells using the anti-MR1 MAbs used in this study (13, 33, 39–41, 50). In earlier mouse studies, MAIT cell hybridomas were selected based on TCR usage, and a small fraction of these MAIT hybridomas were found to be activated by MR1-overexpressing cell lines in a manner blockable by MAbs to MR1 or TCR (13, 39, 56). More recently, mouse MAIT cells from Vα19 TCR-transgenic mice as well as serologically isolated polyclonal human MAIT cells were found to express activation markers after culture with bacterium-infected cells in a manner that was also blockable with anti-MR1 MAb (50). In addition, numerous CD8$^+$ human MAIT cell clones with activity against cells infected with *M. tuberculosis* and other bacteria were found to be blocked by anti-MR1 MAb (33). Although the above-mentioned studies provide compelling evidence for the occurrence of the MR1-dependent activation of MAIT cells, its overall importance relative to noncognate activa-

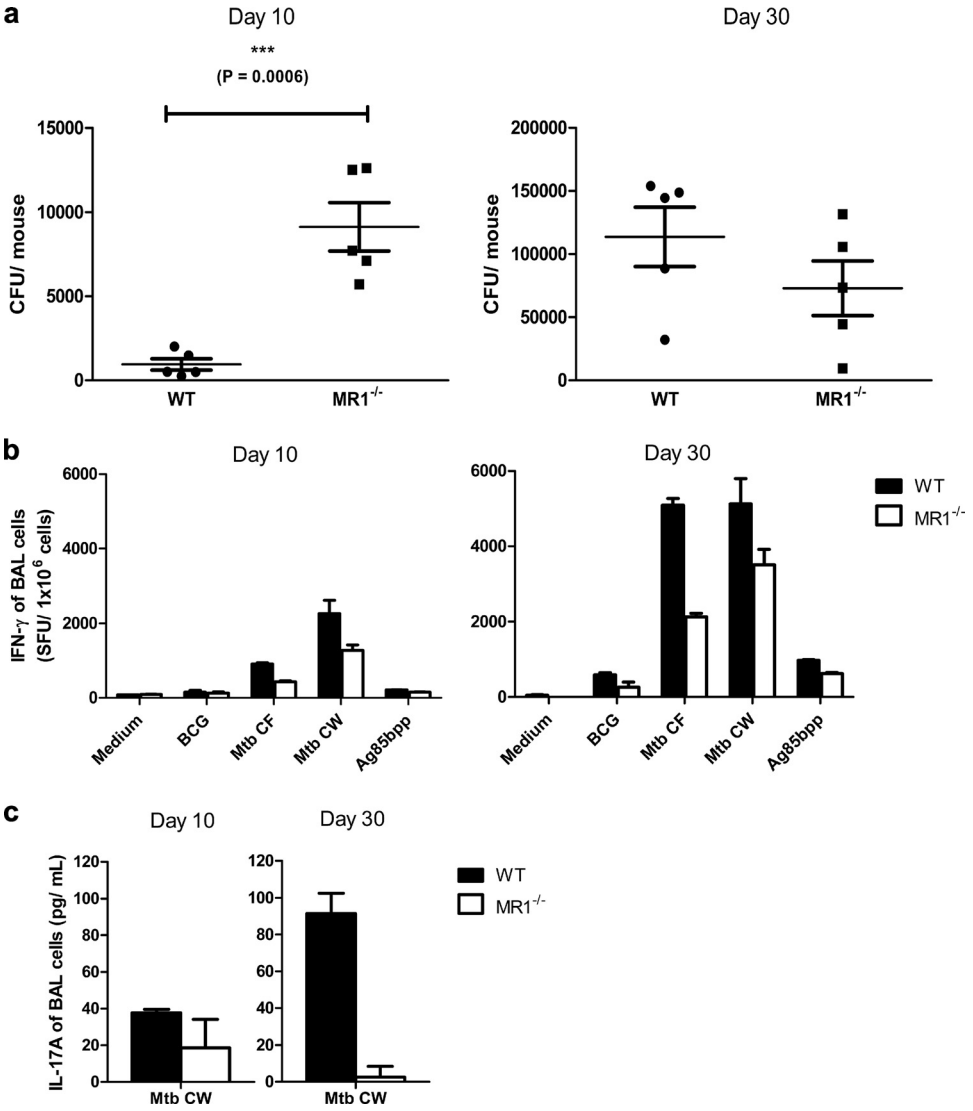

FIG 8 MR1-restricted MAIT cells play a unique role in providing protective immunity against mycobacterial infection *in vivo*. (a) The bacterial burden in WT and MR1$^{-/-}$ mice in the low-dose aerosol infection of *M. bovis* BCG. WT and MR1$^{-/-}$ mice were infected with *M. bovis* BCG via the aerosol route. Each mouse received 100 to 300 infectious bacilli in lung. Ten and 30 days after infection, lung tissues were harvested to determine the bacterial burden. Results are given as the mean ± SEM of five mice per group. Each dot represents the CFU count for one mouse. The difference in bacterial burden between WT and MR1$^{-/-}$ mice at day 10 postinfection is statistically significant as analyzed by Student's *t* test ($P = 0.0008$). Data are results from one of two independent experiments. (b) IFN-γ responses of WT and MR1$^{-/-}$ mice to intranasal infection with *M. bovis* BCG. BAL cells were obtained from WT and MR1$^{-/-}$ mice on day 10 and day 30 after intranasal infection of *M. bovis* BCG (10^7 CFU per mouse). The frequency of IFN-γ-producing cells in the BAL cells was measured by ELISPOT assay. BAL cells from the same genotype were pooled and stimulated with *M. bovis* BCG (MOI = 3) and various *M. tuberculosis*-derived antigens: culture filtrate (CF, 10 μg/ml), cell wall (CW, 50 μg/ml), and an Ag85b peptide pool (Ag85bpp, 2 μg/ml). The numbers of IFN-γ SFUs per one million BAL cells were assessed. Results are means ± SEM from triplicate assays of each set of culture conditions. Data are results from one of two independent experiments. (c) IL-17A production in the supernatant of BAL cells stimulated by the CW fraction of *M. tuberculosis ex vivo*. Results are means ± SEM from triplicate assays. Data are results from one of two independent experiments.

tion is difficult to assess without a better definition of functional heterogeneity within polyclonal MAIT cells.

As a notable parallel, the studies of iNKT cells provide a precedent for how innate T cells bearing limited TCR repertoires employ multiple mechanisms to control microbial infections (6, 8, 74). More specifically, iNKT cells react to microbial infections through at least three activation pathways: microbial antigen-driven, cytokine- and self-antigen-driven, and cytokine-driven pathways (8, 74). While microbial and self lipid antigens detected by iNKT cells have been identified (7, 8, 48, 74), recent studies show that the dominant IL-12 signal from APCs during microbial infection occurs independently of whether microbes express CD1d-presented antigens (9, 15). The IL-12 signal is thought to be an essential factor that enables iNKT cells to respond to a wide variety of microbes with limited TCR repertoires (8, 74). In the case of self-ligand recognition, IL-12 can provide costimulation to amplify a weak TCR-mediated signal (8, 9, 74). Moreover, during viral infection, cytokines, including IL-12 and IL-18, dominate the iNKT cell activation, while CD1d presentation is not required (shown by the use of CD1d$^{-/-}$ mice and/or anti-CD1d MAb)

(75). The constitutive expression of high levels of IL-12 receptor on iNKT cells provides an explanation for the immediate response of iNKT cells to cytokine-driven activation (9). It is likely that, since they share many innate-type features with iNKT cells, MAIT cells are also equipped for rapid responses using cognate and noncognate pathways of activation.

By bridging innate and adaptive immune responses, innate-type T cells are critical for providing the first-line protection against infection (65). In this role, different innate lymphocyte populations share redundant functions. For example, NK cells, γδ T cells, and iNKT cells contribute to innate IFN-γ and/or IL-17A production, but mice lacking functional NK cells, γδ T cells, or iNKT cells are not more susceptible to mycobacterial infection (24, 27, 31, 43, 49, 52, 69). Although MR1$^{-/-}$ mice have normal development of these other innate lymphocytes (72), we show here that MR1$^{-/-}$ mice are defective in the early control of mycobacterial infection. The inability of MR1$^{-/-}$ mice to elicit a protective immune response is likely due to the defective IFN-γ and IL-17A production in the absence of MAIT cells. These findings demonstrate that MAIT cells have a nonredundant role as innate T cells. The infection model of mycobacteria has several advantages to define innate immune responses, including a lengthy doubling time of mycobacteria after lung infection and a slowly developing adaptive immune response (17).

Our *in vivo* findings supporting a role of MAIT cells in mycobacterial infection are congruent with published observations that MAIT cells are activated *in vitro* by mycobacterium-infected cells (33, 50). Similar to our findings, Georgel et al. showed that MR1$^{-/-}$ mice infected intraperitoneally with *K. pneumoniae* failed to control bacterial burden, and serum cytokine analysis of the infected MR1$^{-/-}$ mice showed a decreased proinflammatory response compared to WT mice (32). Again, congruent with these finding, *in vitro* studies showed that MAIT cells displayed activation markers after coculture with cells infected with *K. pneumoniae* (50). Curiously, however, while MAIT cells also showed strong *in vitro* reactivity to *E. coli*, another member of the family *Enterobacteriaceae* (50), MR1$^{-/-}$ mice were not defective in controlling *E. coli* infection via the intraperitoneal route (32). Similarly, cells infected with *M. abscessus* activated MAIT cells *in vitro*, but MR1$^{-/-}$ mice were not defective in controlling bacterial growth *in vivo* (50). However, *Mr1*$^{+/+}$ mice, in which all T cells expressed the transgene of invariant TCRα chain of MAIT cells, had lower bacterial loads after infection with *E. coli* or *M. abscessus* than *Mr1*$^{-/-}$ mice with T cells expressing the same transgenes (50). This finding suggested that overexpression of MAIT cells may be required to detect their *in vivo* functions. However, our report and the report by Georgel et al. (32) would suggest otherwise. Thus, although MAIT cells are activated by diverse microbes *in vitro*, we speculate that their *in vivo* roles are most prominent when infections are introduced into mucosal tissues, when bacterial replication *in vivo* allows a window to monitor innate effector function, and when appropriate innate effector functions are monitored.

Recent findings and speculations of the importance of MAIT cells raise the question of why they were not detected sooner. Indeed, MAIT cells were likely a factor in previously reported findings comparing β_2-microglobulin (β_2m)-deficient mice and CD1d-, H2-M3-, or MHC class Ia-deficient mice (14, 23, 30, 70). Surface expression of MR1 requires β_2m assembly (56), and β_2m$^{-/-}$ mice have been shown to be more susceptible to infections with *K. pneumoniae* (14) and *M. tuberculosis* (23) than mice lacking Kb- and Db-restricted CD8$^+$ T cells or CD1d-restricted iNKT cells. MAIT may also have been involved in the previous studies of nonclassical T cells (either CD8$^+$ or CD4/CD8 DN population) in the murine models of bacterial infection. CD8$^+$ T cells presumably restricted by an undefined class Ib molecule represented a high percentage of IFN-γ producing cells in the lung of Kb$^{-/-}$ Db$^{-/-}$ mice after aerosol infection with *M. tuberculosis* (76). A comparative study using Kb$^{-/-}$ Db$^{-/-}$ and Kb$^{-/-}$ Db$^{-/-}$ M3$^{-/-}$ showed that non-H2-M3 MHC class Ib-restricted CD8$^+$ T cells express activated surface markers, exhibit cytotoxicity, and produce cytokines, including IFN-γ and IL-17A, in response to *L. monocytogenes* infection (11). Although this response was not blocked by anti-MR1 MAb, based on our findings this could be due to non-MR1-dependent activation of MAIT cells. Moreover, it is possible that the role of the MAIT cells in the CD4/CD8 DN T-cell population is overlooked in the studies focusing on CD8$^+$ T cells in Kb$^{-/-}$ Db$^{-/-}$ or Kb$^{-/-}$ Db$^{-/-}$ M3$^{-/-}$ mice (11, 76). In fact, a rare population of CD4$^-$ CD8$^-$ NK1.1$^-$ αβ T cells in WT mice was shown to inhibit the intracellular growth of *M. tuberculosis* and *Francisella tularensis* (LVS) (18, 19), and these CD4/CD8 DN T cells secreted high levels of IFN-γ and IL-17A in response to *F. tularensis* LVS infection (19). These parallels between previous studies and recently defined features of MAIT cells support the notion that MAIT cells are key innate effectors against infection by diverse microbes.

To our knowledge, this study is the first to investigate the *in vivo* role of MAIT cells in a mucosal infection model. Evidence is provided that MAIT cells have an innate effector function and play a nonredundant role in the immune system during bacterial infection. Mechanistically, we show that MAIT cells control bacterial growth and produce proinflammatory cytokines. Further characterization of the mechanisms by which MR1 and MAIT cells are involved in the induction of protective immunity may help in the future design of vaccines against bacterial infection.

ACKNOWLEDGMENTS

This work was support by grants from NIH (AI046553), the Midwest Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research, and McDonnell International Scholars Academy (Washington University in St. Louis). We received *M. tuberculosis* culture filtrate proteins and cell wall fraction as part of NIH, NIAID, contract no. HHSN266200400091C, entitled "Tuberculosis Vaccine Testing and Research Materials," which was awarded to Colorado State University.

We thank Susan Gilfillan for providing Vα19i transgenic mice and Robert Schreiber for providing anti-IFN-γ MAb. We thank Isaac Sakala for technical support and Janet Connolly for reviewing the manuscript.

REFERENCES

1. **Andersen P, et al.** 1992. Identification of immunodominant antigens during infection with Mycobacterium tuberculosis. Scand. J. Immunol. **36**:823–831.
2. **Annibali V, et al.** 2011. CD161(high)CD8+T cells bear pathogenetic potential in multiple sclerosis. Brain **134**:542–554.
3. **Berrington WR, Hawn TR.** 2007. Mycobacterium tuberculosis, macrophages, and the innate immune response: does common variation matter? Immunol. Rev. **219**:167–186.
4. **Berzins SP, Smyth MJ, Baxter AG.** 2011. Presumed guilty: natural killer T cell defects and human disease. Nat. Rev. Immunol. **11**:131–142.
5. **Billerbeck E, et al.** 2010. Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. Proc. Natl. Acad. Sci. U. S. A. **107**:3006–3011.
6. **Borg NA, et al.** 2007. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. Nature **448**:44–49.

7. **Brennan PJ, et al.** 2011. Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals. Nat. Immunol. **12**:1202–1211.
8. **Brigl M, Brenner MB.** 2010. How invariant natural killer T cells respond to infection by recognizing microbial or endogenous lipid antigens. Semin. Immunol. **22**:79–86.
9. **Brigl M, et al.** 2011. Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. J. Exp. Med. **208**:1163–1177.
10. **Chiba A, et al.** 2012. Mucosal-associated invariant T cells promote inflammation and exacerbate disease in murine models of arthritis. Arthritis Rheum. **64**:153–161.
11. **Cho H, Choi HJ, Xu H, Felio K, Wang CR.** 2011. Nonconventional CD8+ T cell responses to Listeria infection in mice lacking MHC class Ia and H2–M3. J. Immunol. **186**:489–498.
12. **Chua WJ, Hansen TH.** 2010. Bacteria, mucosal-associated invariant T cells and MR1. Immunol. Cell Biol. **88**:767–769.
13. **Chua WJ, et al.** 2011. Endogenous MHC-related protein 1 is transiently expressed on the plasma membrane in a conformation that activates mucosal-associated invariant T cells. J. Immunol. **186**:4744–4750.
14. **Cogen AL, Moore TA.** 2009. Beta2-microglobulin-dependent bacterial clearance and survival during murine Klebsiella pneumoniae bacteremia. Infect. Immun. **77**:360–366.
15. **Cohen NR, et al.** 2011. Innate recognition of cell wall beta-glucans drives invariant natural killer T cell responses against fungi. Cell Host Microbe **10**:437–450.
16. **Cooper AM, et al.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. **178**:2243–2247.
17. **Cooper AM, Khader SA.** 2008. The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. Immunol. Rev. **226**:191–204.
18. **Cowley SC, et al.** 2005. CD4-CD8- T cells control intracellular bacterial infections both in vitro and in vivo. J. Exp. Med. **202**:309–319.
19. **Cowley SC, Meierovics AI, Frelinger JA, Iwakura Y, Elkins KL.** 2010. Lung CD4-CD8- double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with Francisella tularensis live vaccine strain. J. Immunol. **184**:5791–5801.
20. **Croxford JL, Miyake S, Huang YY, Shimamura M, Yamamura T.** 2006. Invariant V(alpha)19i T cells regulate autoimmune inflammation. Nat. Immunol. **7**:987–994.
21. **Cua DJ, Tato CM.** 2010. Innate IL-17-producing cells: the sentinels of the immune system. Nat. Rev. Immunol. **10**:479–489.
22. **Curtis MM, Way SS.** 2009. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. Immunology **126**:177–185.
23. **D'Souza CD, et al.** 2000. A novel nonclassic beta2-microglobulin-restricted mechanism influencing early lymphocyte accumulation and subsequent resistance to tuberculosis in the lung. Am. J. Respir. Cell Mol. Biol. **23**:188–193.
24. **D'Souza CD, et al.** 1997. An anti-inflammatory role for gamma delta T lymphocytes in acquired immunity to Mycobacterium tuberculosis. J. Immunol. **158**:1217–1221.
25. **Dusseaux M, et al.** 2011. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. Blood **117**:1250–1259.
26. **Elkins KL, Cowley SC, Conlan JW.** 2011. Measurement of macrophage-mediated killing of intracellular bacteria, including Francisella and mycobacteria. Curr. Protoc. Immunol. **93**:14.25.1–14.25.13.
27. **Feng CG, et al.** 2006. NK cell-derived IFN-gamma differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with Mycobacterium tuberculosis. J. Immunol. **177**:7086–7093.
28. **Flesch IE, Kaufmann SH.** 1991. Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. Infect. Immun. **59**:3213–3218.
29. **Flynn JL, et al.** 1993. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J. Exp. Med. **178**:2249–2254.
30. **Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR.** 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. Proc. Natl. Acad. Sci. U. S. A. **89**:12013–12017.
31. **Gansert JL, et al.** 2003. Human NKT cells express granulysin and exhibit antimycobacterial activity. J. Immunol. **170**:3154–3161.
32. **Georgel P, Radosavljevic M, Macquin C, Bahram S.** 2011. The non-conventional MHC class I MR1 molecule controls infection by Klebsiella pneumoniae in mice. Mol. Immunol. **48**:769–775.
33. **Gold MC, et al.** 2010. Human mucosal associated invariant T cells detect bacterially infected cells. PLoS Biol. **8**:e1000407. doi:10.1371/journal.pbio.1000407.
34. **Gold MC, et al.** 2012. Human thymic MR1-restricted MAIT cells are innate pathogen-reactive effectors that adapt following thymic egress. Mucosal Immunol. [Epub ahead of print.] doi:10.1038/mi.2012.45.
35. **Goldfinch N, et al.** 2010. Conservation of mucosal associated invariant T (MAIT) cells and the MR1 restriction element in ruminants, and abundance of MAIT cells in spleen. Vet. Res. **41**:62.
36. **Gopal R, et al.** 2012. IL-23-dependent IL-17 drives Th1-cell responses following Mycobacterium bovis BCG vaccination. Eur. J. Immunol. **42**:364–373.
37. **Green LC, et al.** 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. **126**:131–138.
38. **Hansen TH, Huang S, Arnold PL, Fremont DH.** 2007. Patterns of nonclassical MHC antigen presentation. Nat. Immunol. **8**:563–568.
39. **Huang S, et al.** 2005. Evidence for MR1 antigen presentation to mucosal-associated invariant T cells. J. Biol. Chem. **280**:21183–21193.
40. **Huang S, et al.** 2008. MR1 uses an endocytic pathway to activate mucosal-associated invariant T cells. J. Exp. Med. **205**:1201–1211.
41. **Huang S, et al.** 2009. MR1 antigen presentation to mucosal-associated invariant T cells was highly conserved in evolution. Proc. Natl. Acad. Sci. U. S. A. **106**:8290–8295.
42. **Jovanovic DV, et al.** 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. J. Immunol. **160**:3513–3521.
43. **Junqueira-Kipnis AP, et al.** 2003. NK cells respond to pulmonary infection with Mycobacterium tuberculosis, but play a minimal role in protection. J. Immunol. **171**:6039–6045.
44. **Kariyone A, et al.** 1999. Identification of amino acid residues of the T-cell epitope of Mycobacterium tuberculosis alpha antigen critical for Vbeta11(+) Th1 cells. Infect. Immun. **67**:4312–4319.
45. **Kawachi I, Maldonado J, Strader C, Gilfillan S.** 2006. MR1-restricted V alpha 19i mucosal-associated invariant T cells are innate T cells in the gut lamina propria that provide a rapid and diverse cytokine response. J. Immunol. **176**:1618–1627.
46. **Khader SA, et al.** 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat. Immunol. **8**:369–377.
47. **Khader SA, Gopal R.** 2010. IL-17 in protective immunity to intracellular pathogens. Virulence **1**:423–427.
48. **Kinjo Y, et al.** 2011. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. Nat. Immunol. **12**:966–974.
49. **Ladel CH, Blum C, Dreher A, Reifenberg K, Kaufmann SH.** 1995. Protective role of gamma/delta T cells and alpha/beta T cells in tuberculosis. Eur. J. Immunol. **25**:2877–2881.
50. **Le Bourhis L, et al.** 2010. Antimicrobial activity of mucosal-associated invariant T cells. Nat. Immunol. **11**:701–708.
51. **Lin Y, et al.** 2009. Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen Francisella tularensis. Immunity **31**:799–810.
52. **Lockhart E, Green AM, Flynn JL.** 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J. Immunol. **177**:4662–4669.
53. **Lozes E, et al.** 1997. Immunogenicity and efficacy of a tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex. Vaccine **15**:830–833.
54. **MacMicking JD, et al.** 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. U. S. A. **94**:5243–5248.
55. **Martin E, et al.** 2009. Stepwise development of MAIT cells in mouse and human. PLoS Biol. **7**:e54. doi:10.1371/journal.pbio.1000054.
56. **Miley MJ, et al.** 2003. Biochemical features of the MHC-related protein 1 consistent with an immunological function. J. Immunol. **170**:6090–6098.
57. **Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG.** 1993. A fluorometric assay for the measurement of nitrite in biological samples. Anal. Biochem. **214**:11–16.
58. **Miyazaki Y, Miyake S, Chiba A, Lantz O, Yamamura T.** 2011. Mucosal-associated invariant T cells regulate Th1 response in multiple sclerosis. Int. Immunol. **23**:529–535.
59. **Nagarajan NA, Kronenberg M.** 2007. Invariant NKT cells amplify the

innate immune response to lipopolysaccharide. J. Immunol. **178**:2706–2713.
60. **Parker S, et al.** 2009. Mousepox in the C57BL/6 strain provides an improved model for evaluating anti-poxvirus therapies. Virology **385**:11–21.
61. **Parra M, et al.** 2009. Development of a murine mycobacterial growth inhibition assay for evaluating vaccines against Mycobacterium tuberculosis. Clin. Vaccine Immunol. **16**:1025–1032.
62. **Pollock JS, et al.** 1991. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc. Natl. Acad. Sci. U. S. A. **88**:10480–10484.
63. **Qian Y, Kang Z, Liu C, Li X.** 2010. IL-17 signaling in host defense and inflammatory diseases. Cell Mol. Immunol. **7**:328–333.
64. **Riegert P, Wanner V, Bahram S.** 1998. Genomics, isoforms, expression, and phylogeny of the MHC class I-related MR1 gene. J. Immunol. **161**:4066–4077.
65. **Rodgers JR, Cook RG.** 2005. MHC class Ib molecules bridge innate and acquired immunity. Nat. Rev. Immunol. **5**:459–471.
66. **Sada-Ovalle I, Chiba A, Gonzales A, Brenner MB, Behar SM.** 2008. Innate invariant NKT cells recognize Mycobacterium tuberculosis-infected macrophages, produce interferon-gamma, and kill intracellular bacteria. PLoS Pathog. **4**:e1000239. doi:10.1371/journal.ppat.1000239.
67. **Schoenborn JR, Wilson CB.** 2007. Regulation of interferon-gamma during innate and adaptive immune responses. Adv. Immunol. **96**:41–101.
68. **Shimamura M, et al.** 2011. Regulation of immunological disorders by invariant Valpha19-Jalpha33 TCR-bearing cells. Immunobiology **216**:374–378.
69. **Skold M, Behar SM.** 2003. Role of CD1d-restricted NKT cells in microbial immunity. Infect. Immun. **71**:5447–5455.
70. **Sousa AO, et al.** 2000. Relative contributions of distinct MHC class I-dependent cell populations in protection to tuberculosis infection in mice. Proc. Natl. Acad. Sci. U. S. A. **97**:4204–4208.
71. **Tilloy F, et al.** 1999. An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. J. Exp. Med. **189**:1907–1921.
72. **Treiner E, et al.** 2003. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. Nature **422**:164–169.
73. **Treiner E, Lantz O.** 2006. CD1d- and MR1-restricted invariant T cells: of mice and men. Curr. Opin. Immunol. **18**:519–526.
74. **Tupin E, Kinjo Y, Kronenberg M.** 2007. The unique role of natural killer T cells in the response to microorganisms. Nat. Rev. Microbiol. **5**:405–417.
75. **Tyznik AJ, et al.** 2008. Cutting edge: the mechanism of invariant NKT cell responses to viral danger signals. J. Immunol. **181**:4452–4456.
76. **Urdahl KB, Liggitt D, Bevan MJ.** 2003. CD8+ T cells accumulate in the lungs of Mycobacterium tuberculosis-infected Kb-/-Db-/- mice, but provide minimal protection. J. Immunol. **170**:1987–1994.
77. **Worku S, Hoft DF.** 2003. Differential effects of control and antigen-specific T cells on intracellular mycobacterial growth. Infect. Immun. **71**:1763–1773.
78. **Yamaguchi H, Hirai M, Kurosawa Y, Hashimoto K.** 1997. A highly conserved major histocompatibility complex class I-related gene in mammals. Biochem. Biophys. Res. Commun. **238**:697–702.
79. **Yamaguchi H, Kurosawa Y, Hashimoto K.** 1998. Expanded genomic organization of conserved mammalian MHC class I-related genes, human MR1 and its murine ortholog. Biochem. Biophys. Res. Commun. **250**:558–564.
80. **Zhang SY, et al.** 2008. Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense. Immunol. Rev. **226**:29–40.